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ORIGINAL RESEARCH

Hormonal regulation of pigment epithelium-derived factor (PEDF) in granulosa cells

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ABSTRACT: Angiogenesis is critical for the development of ovarian follicles. Blood vessels are abrogated from the follicle until ovulation, when they invade it to support the developing corpus luteum. Granulosa cells are known to secrete anti-angiogenic factors that shield against premature vascularization; however, their molecular identity is yet to be defined. In this study we address the physiological role of pigment epithelium-derived factor (PEDF), a well-known angiogenic inhibitor, in granulosa cells. We have shown that human and mouse primary granulosa cells express and secrete PEDF, and characterized its hormonal regulation. Stimulation of granulosa cells with increasing doses of estrogen caused a gradual decrease in the PEDF secretion, while stimulation with progesterone caused an abrupt decrease in its secretion. Moreover, We have shown, by time- and dose–response experiments, that the secreted PEDF and vascular endothelial growth factor (VEGF) were inversely regulated by hCG; namely, PEDF level was nearly undetectable under high doses of hCG, while VEGF level was significantly elevated. The anti-angiogenic nature of the PEDF secreted from granulosa cells was examined by migration, proliferation and tube formation assays in cultures of human umbilical vein endothelial cells. Depleting PEDF from primary granulosa cells conditioned media accelerated endothelial cells proliferation, migration and tube formation. Collectively, the dynamic expression of PEDF that inversely portrays VEGF expression may imply its putative role as a physiological negative regulator of follicular angiogenesis.

Key words: granulosa / hormones / PEDF / angiogenesis / VEGF / ovary

Introduction

The female reproductive organs (i.e. ovaries and uterus), unlike any other organ, undergo cyclic angiogenesis that is critical for their optimal function (Reynolds *et al.*, 2002). During folliculogenesis, the primordial and primary follicles are deprived of an autonomous blood capillary network and receive nutrients and oxygen by passive diffusion from the adjacent stromal blood vessels. The vascular sheath that develops around each follicle at later stages of folliculogenesis is restrained by the follicular basal membrane while communicating only with the theca layer; leaving the granulosa cell layer avascular until after ovulation (Cavender and Murdoch, 1988). Thus, a regulatory mechanism that prevents the penetration of blood vessels into the follicles until ovulation is mandatory. Several studies indicate the existence of such mechanism; though theca cells conditioned culture medium was shown to stimulate proliferation of endothelial cells, regardless of the developmental stage of the follicle (Redmer and Reynolds, 1996), this was not the case for granulosa cells conditioned culture medium. The effect of granulosa cells on the migration and proliferation of endothelial cells, depended on their origin: those derived from follicles at the follicular phase had an inhibitory effect, whereas those derived from follicles just prior to ovulation, on the verge of becoming part of the highly vascular corpora lutea (CL; Fraser, 2006), had already acquired a stimulatory effect (Redmer and Reynolds, 1996; Gruemmer *et al.*, 2005).

While extensive research was invested in characterizing vascular endothelial growth factor (VEGF) as one of the main ovarian pro-angiogenic factors active in the follicle, the nature of the

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physiological anti-angiogenic factor of the ovary is still debatable in the literature in spite of more than 70 years of research (Fevold, 1941; Fraser, 2006).

Pigment epithelium-derived factor (PEDF) is a secreted 50-kDa glycoprotein that belongs to the non-inhibitory members of the serine protease inhibitors (serpin) superfamily. PEDF undergoes several post-translational modifications under different cellular conditions including N-glycosylation and phosphorylation (Simonovic *et al.*, 2001; Lertsburapa and De Vries, 2004; Maik-Rachline *et al.*, 2005; Farkas *et al.*, 2009; Konson *et al.*, 2010; Jia *et al.*, 2011). PEDF was described as a natural angiogenesis inhibitor with neurotrophic and immune-modulating properties (Dawson *et al.*, 1999). The anti-angiogenic effect of PEDF was extensively investigated in the eye, demonstrating its role in decreasing abnormal neovascularization, mainly by inhibiting the stimulatory activity of several strong pro-angiogenic factors, such as VEGF (Stellmach *et al.*, 2001). However, the mechanisms underlying most of these events have not been completely elucidated and it appears that PEDF acts via multiple high-affinity ligands and cell receptors (Manalo *et al.*, 2011).

PEDF was found to be widely expressed in a variety of human body tissues, including the ovaries, as demonstrated by multi-tissue northern blot assays of various fetal and adult human tissues (Tombran-Tink *et al.*, 1996). While silencing PEDF was demonstrated to be of relevance to ovarian surface epithelium carcinogenesis (Cheung *et al.*, 2006), there is no evidence, at present, for a physiological function of PEDF in the ovary; particularly in granulosa cells. The aim of the current study was to characterize the expression of PEDF in granulosa cells, its physiological regulation and its negative effect on angiogenesis.

Materials and Methods

Reagents

The following reagents were used: pregnant mare serum gonadotrophin (PMSG) (Syncro-part, Sanofi, Paris, France), human chorionic gonadotrophin (hCG), 17-beta-estradiol, progesterone and M2 medium (Sigma, St Louis, MO, USA), Dulbecco's modified Eagle's medium /Ham F12 1:1 (DMEM-F12), Dulbecco's PBS (DPBS), penicillin and streptomycin (Biological Industries, Beit-Ha'emek, Israel), endothelial cells growth medium (EGM; Lonza, Basel, Switzerland), fetal bovine serum (FBS; Invitrogen, Grand Island, NY, USA), Hoechst 3342 (Sigma). Primary antibodies: anti-VEGF (ab1316; Abcam, Cambridge, UK), anti-PEDF (sc-25594; Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-actin (MAB1501; Millipore, Temecula, CA, USA). Secondary antibodies: Cy3-conjugated monoclonal antibodies; horse-radish peroxidase (HRP)-conjugated monoclonal and polyclonal antibodies (Jackson Immunoresearch, PA, USA), rabbit Alexa Flour488-conjugated antibodies (Cell signaling technology, MA, USA).

Animals

ICR female mice (Harlan Laboratories, Jerusalem, Israel) were housed in air conditioned, light-controlled animal facilities of the Sackler Faculty of Medicine in Tel-Aviv University. Animal care was in accordance with institutional guidelines and was approved by the Institutional Animal Care and Use Committee.

Cell cultures

Primary mouse granulosa cells were isolated from ovaries of estradiol (E_2)-primed (3 consecutive daily injections of 0.1 ml of 5.7 mg/ml 17-beta-estradiol), 27-day-old mice. The ovaries were incubated in hypertonic sucrose/EGTA medium in order to reduce stress, before they were put into DMEM-F12 medium in the presence of indomethacin (10 μM; Sigma) in order to reduce the production of prostaglandins and needle pricked. Isolated granulosa cells were plated onto serum-coated, 24-well plates (1 ovary/well; Nunc, Copenhagen, Denmark; Orly *et al.*, 1996). Primary human granulosa cells were obtained from 23 women, 22–38 years of age, undergoing IVF treatments (Helsinki IRB approval 167/09*1, Assaf Harofeh Medical Centre, Israel) due to male factor infertility. Patients were treated according to the long protocol guidelines. Granulosa cells were isolated from aspirated follicular fluids after oocytes retrieval. The follicular fluid was centrifuged at 300 g for 5 min at room temperature. The resulting pellets were re-suspended in 10 mM Tris, 0.84% NH_4Cl, pH 7.4, to cause lysis of blood cells (15 min shaking at 37°C) and were washed several times in phosphate-buffered saline (PBS) to eliminate debris. Cells were plated in DMEM-F12, supplemented with penicillin (100 IU/ml), streptomycin (100 mg/ml) and 10% FBS. Cells were counted before seeding in order to reach equal confluence and make sure there is no contamination by leukocytes. Cells were washed every 24 h with PBS and cultured in hormone-free medium as described previously (Breckwoldt *et al.*, 1996; Sasson and Amsterdam, 2002). Cells were serum-starved (0.1% FBS) for 8 h before stimulation.

Human umbilical vein endothelial cells (HUVECs) were obtained from the American Type Culture Collection (ATCC) and cultured in EGM-2 medium. All cells were cultured at 37°C; 5% CO_2.

Western blot analysis

Proteins from granulosa cells or from oocytes isolated at the germinal vesicle (GV) stage (250–350 oocytes) were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE; 10%; Bio-Rad, Israel) and transferred onto nitrocellulose membranes (Whatman GmbH, Germany) in a mini-tank transfer unit (TE 22, Amersham, UK). Approximate molecular masses were determined by comparison with the migration of pre-stained protein standards (Bio-Rad). Blots were blocked for 1 h in TBST (10 mM Tris–HCl, pH 8.0, 150 mM NaCl, 0.05% Tween 20) containing 5% skim milk (Alba, NY, USA) followed by an over-night incubation at 4°C with primary antibodies. Blots were washed three times in TBST and incubated for 1 h at room temperature with HRP-conjugated secondary antibodies. Immunoreactive bands were visualized by enhanced chemiluminescence (Thermo Scientific, IL, USA) according to the manufacturer's guidelines. The intensity of the protein bands was quantified by ImageJ sportswear (NIH).

Immunohistochemistry

Paraffin-embedded sections of ovaries from 7-week-old mice were deparaffinized, microwave heated while being subjected to an antigen retrieval agent (H-3300, Vector Laboratories, Inc., Burlingame, CA, USA), cooled on ice to room temperature, rinsed in PBS, incubated for 1 h with PBSTg (0.2% Tween and gelatin in PBS), washed with PBS, blocked for 10 min in blocking solution (Cell Marque Corporation, CA, USA) and incubated overnight with anti-PEDF antibody. At the following day, sections were washed in PBSTg and PBS before and after applying the appropriate secondary antibodies together with a nuclear marker (Hoechst 3342), rinsed, mounted with moviol (Sigma), visualized and photographed by a Leica laser confocal microscope (SP5 Wetzlar, Germany) that was calibrated to a secondary-only control.

Immunofluorescence

Ovarian and oviductal oocytes of 7-week-old ICR mice were isolated into M2 medium (Sigma). Zonae pelucidae were removed by a brief exposure to alpha-chymotrypsin (50 μg/ml in 1 mM HCl; Sigma), fixed by 3% paraformaldehyde (Merck, Gibbstown, NJ, USA), washed in blocking solution (3% FBS in DPBS), permeabilized (10 min, 0.05% Nonidet P-40; Sigma), incubated for 1.5 h in the presence of anti-PEDF antibody, washed in blocking solution and incubated for an additional 1 h with Cy3-conjugated secondary antibody (Levi *et al.*, 2010). Stained oocytes were visualized and photographed by a Leica laser confocal microscope.

Protein precipitation

Culture media of starved cells (16 h, 0.1%FBS) were collected and centrifuged after addition of 10% (v/v) trichloroacetic acid (TCA; Sigma) for 16 h at −20°C. Pellets were washed with ice-cold acetone and re-suspended in SDS-PAGE loading buffer.

PEDF production

Human recombinant PEDF (NM_002615.4) was expressed in *E. coli* BL21. Bacteria were allowed to grow at 30°C to OD_{600nm} of 0.5–0.6, induced for 4–5 h by 0.5 mmol/l isopropyl-L-thio-β-D-galactopyranoside, centrifuged and their pellets were lysed. Recombinant proteins were purified by ion metal affinity chromatography with Ni-NTA His-Bind resin (Merck KGaA, Darmstadt, Germany) according to the manufacturer's protocol. Eluted fractions were resolved by SDS-PAGE followed by GelCode (Blue Stain Reagent, Thermo scientific, USA) or western blotting with a specific anti-PEDF antibody. Eluates with >90% purity were dialyzed against buffer TRIS (pH 10.0; Konson, Pradeep and Seger, 2010).

Cell proliferation assay

HUVECs were plated on 24-well plates (1.5×10^4cells/well) and cultured for 24 h. Culture media in some of the wells were replaced for the next 72 h with lyophilized conditioned media of the first-day or fifth-day of culture of primary human granulosa-lutein cells and the HUVECs were re-suspended in EGM-2. Culture media in other wells were replaced for 24 h with fifth-day conditioned media and immunoprecipitated with PEDF antibodies or with IgG (diluted to 60% in DMEM-F12). Following incubation, cells were counted by Coulter Counter (Beckman Coulter, CA, USA).

Capillary-like tube formation assay

In vitro capillary-like tube formation of HUVECs was assessed as follows: the surface of 24-well plates was coated with Matrigel cultrex® basement membrane (50 μl/well; 10 mg/ml; R&D Systems, MN, USA). HUVECs (3×10^4 cells/well) were challenged for 6 h with conditioned media of primary human granulosa-lutein cells (37°C; 5% CO_2). Wells were imaged by Nikon TE2000E inverted microscope (4× objective; bright field) integrated with a Nikon DS5 cooled CCD camera.

Migration assay

Cell migration assay was performed using modified 8-μm Boyden chambers (Transwell-Costar Corp., Cambridge, MA, USA) coated with 10 μg/ml fibronectin (Biological Industries). HUVECs (1.5×10^5cells/well) were cultured in DMEM-F12 serum-free medium for 2 h at the upper Boyden chambers. Conditioned medium was added to the lower chambers and HUVECs were allowed to migrate for 4 h before fixation and staining (Hema-3 Stain System; Fisher Diagnostics, Houston, TX, USA). The number of migrated cells per membrane was captured using a bright-field microscope connected to a spot digital camera (Diagnostic Instruments, Sterling Heights, MI, USA) and counted using the NIH ImageJ processing and analysis software. The degree of migration towards medium containing 10% FBS was normalized as 100%.

RNA isolation, reverse transcription, PCR and real-time PCR (qPCR)

Total RNA was isolated from various tissues (ovaries, eyes) or from granulosa cells, using Trizol reagent according to the manufacturer's instructions, and quantified with the Nano-Drop spectrophotometer (ND-1000; Thermo Scientific). First-strand cDNA was created by RT (Maxima TM Reverse transcriptase, Fermentas, MA, USA) from a total of 1 μg RNA, using oligo-dt primers (Fermentas). RNA was also extracted from batches of 100 oocytes and reverse transcribed it into cDNA, using Cells-to-ct (ambion, Grand Island, NY USA). DNA was amplified using 1 μl RT reaction and 50 pmol gene-specific primers in ReadyMix™ mixture (Sigma). PCR products were separated by 1.5% agarose gel electrophoresis and visualized by ethidium bromide staining. Changes in the level of expression of mRNA were detected by SYBR green reagent (SYBR® Green PCR Master Mix, ABI, Carlsbad, CA, USA) along with 15 ng cDNA and specific primers, on a StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA).

Primers for PCR

Actin (253 bp)	Mouse	Forward: 5′CATCCGTAAAGACCTCTATGCCAAC3 Reverse: 5′CAAAGAAAGGGTGTAAAACGCAGC3′
GPDH (536 bp)	Mouse/rat	Forward: 5′GTGAAGGTCGGTGTGAACGG3′ Reverse: 5′GTGATGGCATGGACTGTGGTC3′
PEDF (533 bp)	Rat	Forward: 5′CATTCACCGGGCTCTCTACTA3′ Reverse: 5′TCAGGGGCAGGAAGAAGATGAT3′
PEDF (372 bp)	Mouse	Forward: 5′TCTCCTTGGCGTGGCTTACTTCAA3′ Reverse: 5′ TGCAGAGACTTGGTAAGTTCGCCT3′
PEDF (73 bp)	Mouse	Forward: 5′CCAAGTCTCTGCAGGACATGAAG3′ Reverse: 5′GGTTTGCCAGTAATCTTGCTG3′

Primers for qPCR

HPRT1	Mouse	Forward: 5′CTCATGGACTGATTATGGACAGGA3′ Reverse: 5′GCAGGTCAGCAAAGAACTTATAGCC3′
PEDF	Mouse	Forward: 5′CCAAGTCTCTGCAGGACATGAAG3′ Reverse: 5′GGTTTGCCAGTAATCTTGCTG3′
VEGF	Mouse	Forward: 5′AGGCTGCTGTAACGATGAAGC3′ Reverse: 5′AGGTTTGATCCGCATGATCTG3′

Statistical analysis

All experiments were performed three to five times. We presented a few chosen representative western blot and immune-staining micrographs. The

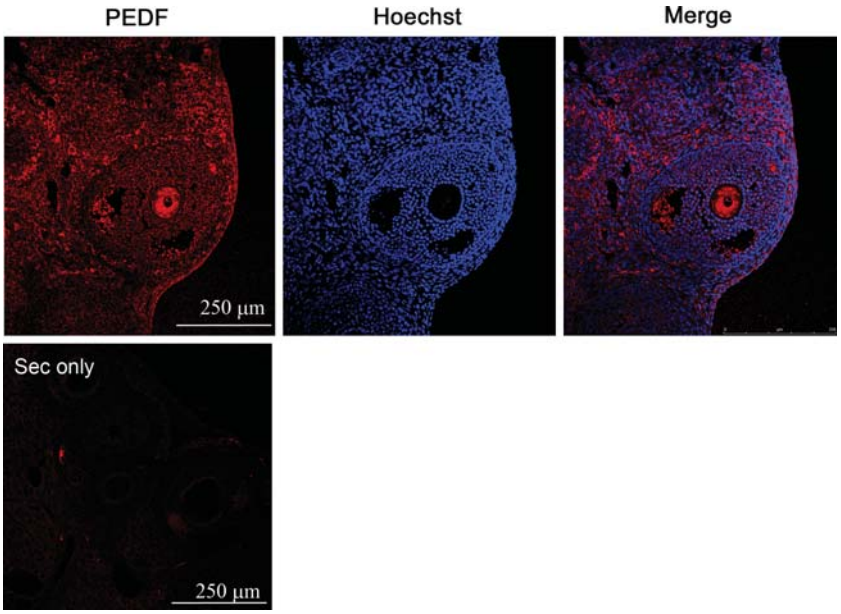

Figure 1 PEDF is expressed in the follicle. A representative histological section of mouse ovary labeled with an anti-PEDF antibody (red) and Hoechst (blue) as a nuclear marker.

graphs represent normally distributed data that are expressed as the mean ± SDV and were evaluated by functional student's *t*-test (two-tailed); $P < 0.05$ was considered statistically significant.

Results

PEDF expression in the ovary

To characterize the role of PEDF as an anti-angiogenic factor in the ovary, we initially followed its expression pattern. Histological sections of mouse ovaries immunostained with an anti-PEDF antibody showed that PEDF is highly expressed in the ovary including granulosa cells, theca cells and oocytes (Fig. 1). Given that PEDF is a secreted glycoprotein, and since the communication between the oocyte and its surrounding granulosa cells is bidirectional (Gilchrist *et al.*, 2008), we evaluated PEDF mRNA and protein in freshly isolated oocytes (Fig. 2) as well as in granulosa cells (Fig. 3).

We were able to demonstrate the expression of PEDF both at the mRNA (Fig. 2A) and protein (Fig. 2B–C) level in ovarian oocytes at the GV stage. In addition, we detected PEDF mRNA in primary mouse granulosa cells using retina and cornea as positive controls (Fig. 3A; Dawson, *et al.*, 1999). We further tested the ability of granulosa cells to express and secrete PEDF protein using various cells sources. PEDF protein was expressed in the cell lysate of both primary mouse and human granulosa cells (Fig. 3B), and also present in abundant quantities in the culture media (Fig. 3C). Since the secreted PEDF is a well-known anti-angiogenic factor (Takenaka *et al.*, 2005), and since we found it to be biosynthesized by and

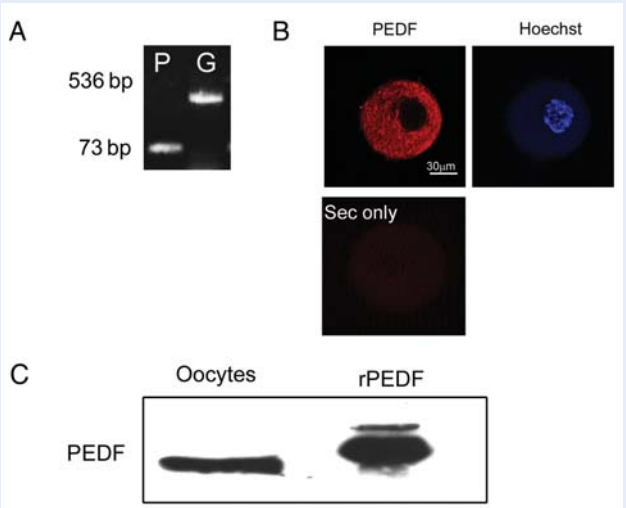

Figure 2 PEDF expression in the oocyte. GV oocytes were isolated from ovarian follicles of mice administered with 5 IU PMSG. (**A**) Autoradiographs of representative PCR analyses (100 oocytes, X35 cycles) demonstrating the expression of PEDF mRNA (P; 73bp) and the endogenous control GAPDH (G; 536 bp). (**B**) A representative immuno-staining micrograph of freshly isolated GV oocyte labeled with anti-PEDF antibody (red) and Hoechst (blue) as a nuclear marker. (**C**) A representative western blot of GV oocytes (350) and recombinant PEDF (rPEDF; control) reacted with anti-PEDF antibody. All experiments (A–C) were repeated at least three times.

secreted from granulosa cells, we propose that PEDF plays an important role in regulating ovarian angiogenesis.

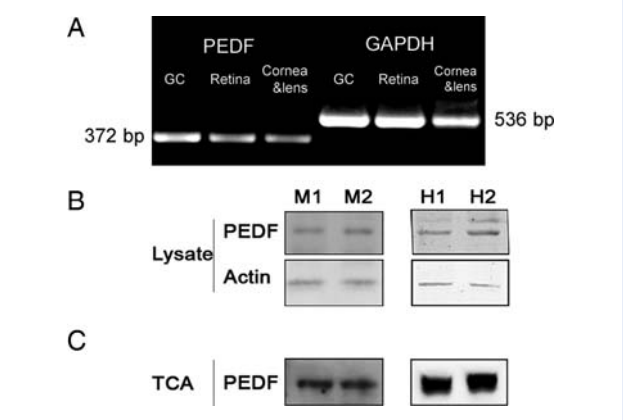

Figure 3 PEDF is produced by granulosa cells. (**A**) Autoradiographs of a representative PCR analysis (×35 cycles) demonstrating the expression of PEDF mRNA in primary mouse granulosa cells (GC) from follicles of 27-day-old ICR mice primed with estrogen for 3 days and cultured for 7 days before mRNA extraction. Mouse retina, cornea and lens served as a positive control. GAPDH primers served as an endogenous control. (**B** and **C**) Western blot analysis of PEDF protein in cultured granulosa cell lysates (B; Lysate; upper panel) and in their corresponding culture media (C; TCA). Primary mouse granulosa cells were obtained from follicles of 27–day-old ICR mice (M1, M2). Primary human granulosa-lutein cells were obtained from follicles of women undergoing IVF treatments (H1, H2). Both primary cells types were cultured for 7 days before lysis. All blots were incubated with anti-PEDF antibody and calibrated with anti-actin antibody (B; lower panel).

Hormonal regulation of PEDF secretion

Steroidal hormones, estrogen (E_2) and progesterone (P4), modulate granulosa cells function and ovarian angiogenesis (Mahesh, 1985; Schaison and Couzinet, 1991). We, therefore, speculated that they will affect the PEDF level. In order to test this hypothesis, primary human granulosa cells were pre-cultured for 1 week in a hormone-free medium (in order to reach quiescence), serum-starved for 8 h and stimulated with increasing concentrations of E_2 (pg/ml) or P4 (ng/ml) for 16 h. We found that the stimulation of human granulosa cells with increasing doses of E_2 caused a gradual decrease in the PEDF secretion (Fig. 4A–B); while, the stimulation with P4 caused a sharp reduction in the PEDF secretion, down to an undetectable level (Fig. 4C–D).

The LH surge triggers ovulation and the development of a new corpus luteum. During the early luteal phase in which the initiation of development of the luteal microvasculature is underway, intense angiogenesis is found as indicated by the high rate of endothelial cell proliferation (Wulff *et al.*, 2001). Therefore, our aim was to evaluate the effect of hCG on PEDF level. In order to do so, primary human granulosa cells were cultured in hormone-free medium (day of seeding is referred to as Day 0). We collected the culture media of the granulosa cells at several time intervals and found that as the time from *in vivo* exposure to hCG elapsed, the cells regained their ability to secrete PEDF (Fig. 5A and B). Given that VEGF is one of the main pro-angiogenic factors in the ovary (Fraser, 2006) and since VEGF and PEDF were shown to be inversely regulated in other organs (Cai *et al.*, 2006), we hypothesized that ovarian PEDF and VEGF are also oppositely regulated, thus allowing the maintenance of coordinated angiogenesis in the ovary (Mahesh, 1985). Therefore, we assessed the levels of VEGF in the same conditioned media and found that opposed to PEDF, VEGF secretion decreased as the time from hCG administration elapsed (Fig. 5A and C). These findings indicate that the expression of PEDF and VEGF in granulosa cells is inversely regulated following hCG stimulation.

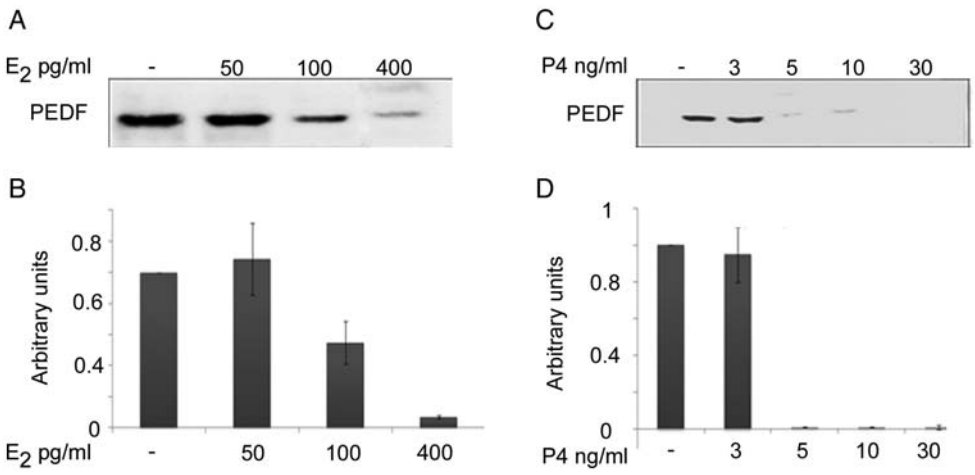

Figure 4 Estrogen and progesterone down-regulate PEDF expression in cultured primary human granulosa-lutein cells. Western blot analyses and their corresponding quantification of PEDF protein from conditioned media of cultured serum-starved primary human granulosa-lutein cells, pre-cultured for 1 week and treated for 16 h with increasing concentrations of estrogen (E_2; **A** and **B**) or progesterone (P; **C** and **D**), both dissolved in ethanol. Control group was treated with the same volume of ethanol. Bars are mean ± SDV.

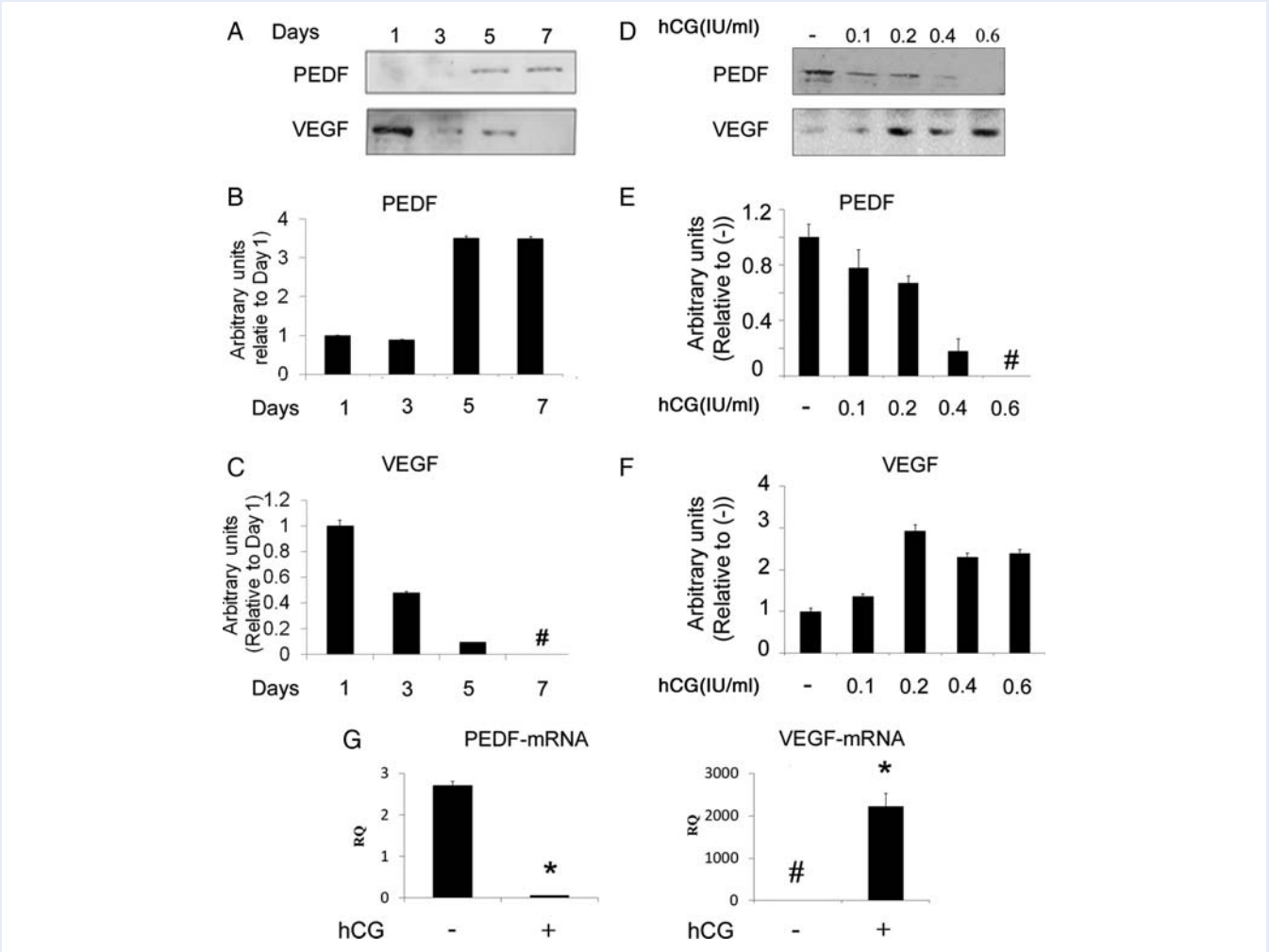

Figure 5 hCG regulates PEDF and VEGF in an opposing manner. Reciprocal expression of PEDF and VEGF in primary human granulosa cells. (**A**) A representative western blot analyses of PEDF and VEGF proteins precipitated from conditioned media of primary human granulosa-lutein cells and (**B** and **C**) their corresponding quantification. Cells were cultured up to 7 days with daily media replacements. Media were collected on culture days 1, 3, 5 and 7 and proteins were precipitated by TCA. (**D**) A representative western blot analyses of PEDF and VEGF proteins precipitated from culture media of serum-starved primary human granulosa cell treated with increasing doses of hCG and (**E** and **F**) their corresponding quantification. Bars in B, C, E, F, are the mean ± SDV of three independent experiments. (#) represents value <0.001. Reciprocal expression of PEDF and VEGF in mice pre- and post-ovulatory primary granulosa cells. (**G**) Graphic representation of qPCR analyses with specific primers for PEDF or VEGF; calibrated with HPRT. mRNA was extracted from primary mouse granulosa cells isolated either from follicles of mice administered with 5 IU PMSG (hCG−) or with 5 IU PMSG and 7 IU hCG (hCG+). Bars are the mean ± SDV of relative quantification (RQ), 6 mice/treatment, *Significantly different from the control value ($P < 0.05$; *t*-test).

Our next aim was to evaluate the *in vitro* effect of hCG stimulation on PEDF and VEGF secretion in 'quiescent' human primary granulosa cells (pre-cultured for a week). We found a reciprocal dose−response effect of hCG on PEDF and VEGF secretion; namely, PEDF secretion decreased significantly concomitantly with an up-regulation of VEGF secretion (Fig. 5D−F).

Finally, we wanted to verify the influence of *in vivo* administration of hCG on the level of PEDF and VEGF in freshly isolated mouse granulosa cells (Fig. 5G), and found that while PEDF mRNA was highly expressed in granulosa cells prior to hCG administration, its post-hCG level was decreased. On the other hand, the level of VEGF mRNA was up-regulated following hCG administration.

PEDF secreted by granulosa cells exerts a potent anti angiogenic effect

Our findings demonstrating the inverse hormonal regulation of PEDF and VEGF led us to examine the anti-angiogenic activity of PEDF, secreted from granulosa cells, on HUVECs functions (Hutchings *et al.*, 2002; Konson *et al.*, 2011). We incubated HUVECs in culture media conditioned by primary human granulosa cells retrieved as described above. The culture media were collected every 24 h for 5 days. We initially compared the ability of HUVECs to proliferate in the presence of granulosa cells conditioned media, collected at the first- or fifth-day of culture (Fig. 6A). The proliferation rate of

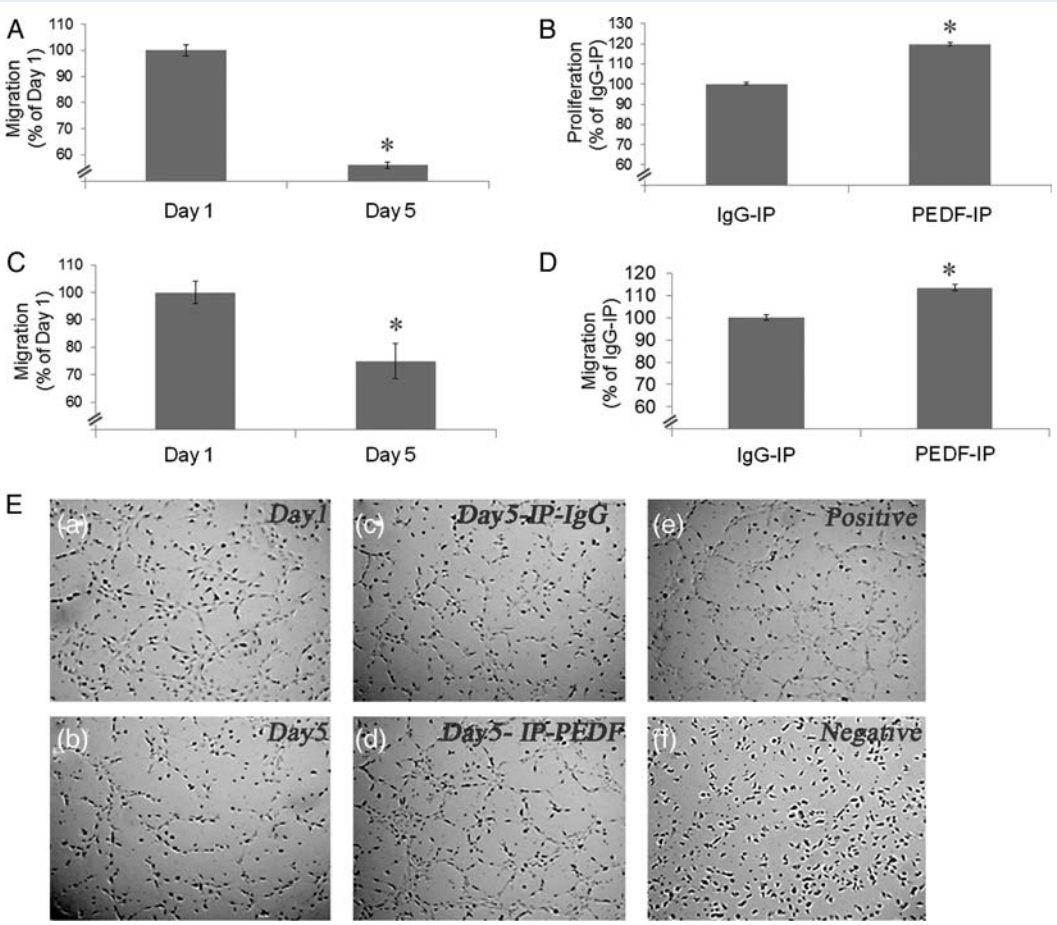

Figure 6 PEDF secreted from granulosa cells inhibits endothelial cells activity. Primary human granulosa-lutein cells were harvested from follicles of women undergoing IVF treatments, 34–36 h after hCG administration. The cells were cultured for 1–5 days with daily media replacements. (**A**) Endothelial cells proliferation. HUVECs were cultured with conditioned media collected at the first- or fifth-day of culture. Bars (mean ± SDV) are the number of HUVECs at the end of the incubation period as percent of naïve control cells (EGM culture media, calibrated to 'Day 1'); *Significantly different from 'Day 1' value ($P < 0.05$; *t*-test). (**B**) PEDF secreted into the culture media affects the proliferation of endothelial cells. HUVECs were cultured for 24 h with fifth-day conditioned medium that was pre-immuno-precipitated with either PEDF (PEDF-IP) or IgG (control; IgG-IP). Bars (mean ± SDV) are the number of HUVECs at the end of the incubation period, as percent of naïve control cells (10% FBS in DMEM-F12); *Significantly different from the IgG-IP value ($P < 0.05$; T-test). (**C**) Endothelial cells migration. HUVECs were seeded in the DMEM-F12 serum-free medium and allowed to migrate toward first-day or fifth-day conditioned-media. Bars (mean ± SDV) are the number of migrated cells at the end of the incubation period, as percent of control (10% FBS in DMEM-F12); *Significantly different from the first-day value ($P < 0.05$; *t*-test). (**D**) PEDF secreted into the culture media affects the migration of endothelial cells. HUVECs were seeded in the DMEM-F12 serum-free medium and allowed to migrate toward fifth-day conditioned medium, immuno-precipitated with either PEDF (PEDF-IP) or IgG (control; IgG-IP). Bars (mean ± SDV) are number of migrated cells at the end of the incubation period, as percent of control (10% FBS in DMEM-F12); *Significantly different from IgG-IP value ($P < 0.05$; *t*-test). (**E**) PEDF secreted into the culture media affects the ability of endothelial cells to form tubular structures. Representative images of capillary-like tube structures of HUVECs seeded on Matrigel following various treatments: a, first-day conditioned medium; b, fifth-day conditioned medium; c, IgG-IP fifth-day conditioned medium; d, PEDF-IP fifth-day conditioned medium; e, positive control (10% FBS in DMEM-F12); f, negative control (no Matrigel).

HUVECs was significantly lower following incubation with fifth-day conditioned medium as compared with first-day conditioned medium (Fig. 6A); in accordance with the inverse expression of VEGF and PEDF at the corresponding culture days (Fig. 5A). In order to evaluate whether this anti-proliferative effect is attributed to PEDF up-regulation, we immuno-precipitated PEDF (PEDF-IP) from the conditioned medium collected at the fifth day, before culturing the HUVECs in it (Fig. 6B). We found that the proliferation rate of HUVECs was significantly higher following incubation with PEDF-IP fifth-day conditioned medium as compared with IgG-IP fifth-day conditioned medium (Fig. 6B).

Furthermore, we evaluate the ability of HUVECs cultured in Boyden chambers in serum-free media to migrate towards first-day or fifth-day conditioned media. We found that the migration rate of HUVECs was significantly lower following incubation with fifth-day conditioned medium as compared with first-day conditioned medium (Fig. 6C).

Similar to the proliferation assay, PEDF-IP fifth-day conditioned medium significantly increased the migration rate of HUVECs as compared with IgG-IP fifth-day conditioned medium (Fig. 6D). Finally, we assessed the effect of various conditioned media on the capability of HUVECs to create capillary-like tube structures. We showed that the incubation of HUVECs in first-day conditioned medium (Fig. 6Ea) as well as in PEDF-IP fifth-day conditioned medium (Fig. 6Ed) induced the formation of significantly more capillary-like networks, as compared with HUVECs cultured in either fifth-day conditioned-medium (Fig. 6Eb) or IgG-IP fifth-day conditioned-medium (Fig. 6Ec).

Altogether, these results demonstrate that PEDF, secreted from granulosa cells, exerts a potent anti-angiogenic activity.

Discussion

Towards ovulation, follicular growth is accompanied by a gradual increase in the production of E_2 by granulosa cells, which peaks at ovulation (Mahesh, 1985; Schaison and Couzinet, 1991). Moreover, following the LH surge there is a rise in P4 level that remains high to support the developing CL and early pregnancy (Stouffer, 2003; Shimizu and Miyamoto, 2007). This process is characterized by a rapid growth of blood vessels into the follicle toward the granulosa cells (Phan *et al.*, 2006). In the current study we demonstrate that PEDF is produced by the ovarian follicle; by both granulosa cells and oocyte. In the current study we chose to focus on expression and regulation of PEDF by granulosa cells. We found that PEDF is secreted by granulosa cells of both rodents and humans, and its expression is hormonally regulated. Increasing doses of E_2 and hCG induced a gradual decrease in PEDF secretion, while stimulation by P4 caused an abrupt decrease in its secretion. We therefore postulate that the effect of PEDF on the follicular vasculature changes according to the hormonal milieu: at the beginning of the cycle, when E_2 level is low, PEDF is robustly expressed by granulosa cells. Towards ovulation, the gradually increasing levels of E_2 are followed by LH surge and by an increase in P4 level; each of them independently reduces PEDF expression level.

VEGF has been demonstrated to be one of the main pro-angiogenic factors in the ovary and its dynamic regulation was well characterized. Stimulation of granulosa cells by hCG as well as by IGFs and hypoxia induced up-regulation of VEGF (Hazzard *et al.*, 1999; Tropea *et al.*, 2006; Taylor *et al.*, 2007) that is cardinal for generation of healthy ovulatory follicles and CL (Distler *et al.*, 2003). In addition to VEGF, other factors such as angiopoietin (1 and 2; (Sugino *et al.*, 2005), leukocytes (Polec *et al.*, 2011) and platelets (Furukawa *et al.*, 2007; Nurden, 2007) contribute to the remodeling of endothelial cells and luteinized granulosa cells in the process of CL formation. In the current study we found that PEDF regulation is hormonally affected inversely to VEGF, further implying a role for PEDF as a negative regulator of ovarian angiogenesis.

On top of its VEGF-dependent anti-angiogenic effect, PEDF is known to be involved in angiogenesis inhibition through several other mechanisms. These include post-translational modifications of PEDF as N-glycosylation and phosphorylation that occur under different conditions (Simonovic *et al.*, 2001; Lertsburapa and De Vries, 2004; Maik-Rachline *et al.*, 2005). PEDF was found to be phosphorylated by CK2 and PKA; it was shown that the differential phosphorylation induces variable effects of PEDF, among them the regulation of angiogenesis (Maik-Rachline and Seger, 2006). However, further research is needed to characterize the post-translational modifications of PEDF within the ovary. Furthermore, a 60 kDa PEDF putative receptor (PEDF-RA; PEDF-R) localized on endothelial cells was recently found to be *involved* in the direct anti-angiogenic effect of PEDF (reviewed by Manalo *et al.*, 2011). Binding of PEDF to the receptor induced endothelial cell apoptosis, while angiogenesis, migration, tumor cell adhesion and proliferation were inhibited.

The dynamic changes in the vasculature of ovarian follicles mandate a delicate balance of pro- and anti-angiogenic factors (Maisonpierre *et al.*, 1997; Tempel *et al.*, 2000; Shang *et al.*, 2001; Greenaway *et al.*, 2005; Gruemmer *et al.*, 2005; Fraser, 2006). The identity of ovarian anti-angiogenic factors has been intensively investigated, suggesting several candidates, among them thrombospondin (TPS; Garside *et al.*, 2010) and hyaluronic acid (Tempel, Gilead and Neeman, 2000). Though TPS, produced and secreted by bovine granulosa cells, is positively regulated by FSH, stimulation by LH had no effect on its expression; suggesting its role is mainly during the follicular phase. Furthermore, although hyaluronic acid exerts an *in vitro* inhibitory effect on endothelial cells activity, this activity is not hormonally regulated.

In conclusion, in the current study we demonstrate that PEDF is produced in granulosa cells and secreted by them, both in rodents and human. The secreted PEDF possesses an anti-angiogenic effect, as demonstrated by *in vitro* inhibition of HUVECs proliferation, migration and tube formation. Imbalanced angiogenesis lies at the core of several fertility-related pathologies such as ovarian hyper-stimulation syndrome and endometriosis (Reynolds *et al.*, 2002; Fainaru *et al.*, 2009), therefore, these findings may confer future potential clinical implications on PEDF.

Authors' roles

D.C. and B.I. developed the concept, designed experiments and prepared the manuscript. D.C. also carried out most of the experiments, data organization and statistical analyses and wrote the manuscript. R.K.K. helped drafting the manuscript. H.G. preformed real-time experiments. AK assisted in collecting mouse oocyte. A.E.B. conducted the angiogenesis assays. R.S.F. participated in designing the angiogenesis assays. R.R. discussed the manuscript. R.S. conceived the study, participated in its design and coordination, helped drafting the manuscript and supervised the study. All authors read and approved the final manuscript.

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Conflict of interest

R.K.K., H.G., A.K., R.S.F. and A.E.B. have nothing to declare. D.C., I.B., R.R. and R.S. are inventors on U.S. Patent/PCT WO2011/058557.

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